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In this proposal we have investigated the hypothesis that overexpression of a novel truncated version of AIB1 ( $\Delta$ 3AIB1) that we have found to be overexpressed in breast cancer is import for tumor development by impacting upon nuclear hormone receptor function. We have examined the hypothesis that the novel AIB1 variant has an altered function that changes its interaction with nuclear receptors such as the estrogen or progesterone receptor. We propose that changes in the level of expression of the novel AIB1 variant will support tumor progression and may well have prognostic significance for breast cancer. We have now determined that  $\Delta$ 3AIB1 is overexpressed relative to the full-length protein in breast cancer tumor samples and cell lines. We have also determined that  $\Delta$ 3AIB1 is a significantly more active coactivator than full-length AIB1 on the estrogen and progesterone receptor (*J. Biol. Chem.* 276, 39736-39741, 2001). In addition, a surprising finding is that overexpression of  $\Delta$ 3AIB1 can also potentiate EGF signaling. This implies that  $\Delta$ 3AIB1 can also drive non-hormone mediated proliferation in breast cancer.

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## Introduction

An area of chromosome 20q, frequently amplified in breast cancer harbors a steroid receptor coactivator (SRC) gene, AIB1 (amplified in breast cancer 1). (1) SRCs are a family of proteins that bind to the liganded receptor and when overexpressed are able to increase the maximal response to steroids such as estrogen and progesterone. (2) The AIB1 gene is amplified in 5-10% of breast cancers and the mRNA is overexpressed in about 60% of tumors. (1) We have also determined that the AIB1 protein is overexpressed in breast cancer by immunocytochemical analysis. (3) The role of AIB1 is further complicated by overexpression studies that demonstrate that AIB1 cannot only potentiate the action of the estrogen and progesterone receptor but also a number of other nuclear receptors in transient transfection over expression assays. (2) Interestingly, some of these receptors such as retinoid and vitamin D<sub>3</sub> have predominantly anti-proliferative and pro-apoptotic roles in breast cancer whereas other receptors such as estrogen, progesterone and thyroid receptor are considered to induce growth by increased proliferation and reduced apoptosis. Thus it is possible to envisage the overexpression of AIB1 as enhancing both proliferative and antiproliferative effects of nuclear receptors. It is also apparent that the effects of the SRC coactivators are not confined to interactions with the nuclear receptor family. The SRC proteins also interact with other cofactors such as the pCAF and CBP/p300 cointegrators (4) that are recipients of signals from a number of cellular signaling pathways. In addition, AIB1 has been described as interacting with p53. (5) Thus, it is entirely possible that AIB1 may have wide ranging effects on cell cycle and proliferation that are independent of its effects on nuclear receptor function.

Another aspect of nuclear coactivators which is particular interesting is the existence of exon splice variants of the SRC-1 and SRC-3 (AIB1) genes that can be predicted from the cDNA cloning data, although the levels of expression and impact of the presence of these alternative splice products on coactivator function is not known (2). For ACTR/AIB1, such putative splice variants can be predicted within the receptor interacting region and in the N-terminal helix-loop-helix-(HLH)-Per-Arnt-Sim (PAS) domain. We have now detected a novel  $\Delta$  exon 3 AIB1 splice variant that is expressed in MCF-7 breast cancer cells as well as in breast tumor tissue. (appendix manuscript) We have determined the exon intron structure of AIB1 from the human genome database and the predicted protein product from this isoform is an N-terminally truncated version of AIB1 that has a deleted HLH domain and most of the PAS region removed. The predicted size of this protein is approximately 125 kDA and a protein with this molecular weight is detected in our Western blot analysis of MCF-7 cells and is translated *in vitro* from the  $\Delta$  exon 3 AIB1 cDNA.

To our knowledge this is the first report of expression of an AIB1 splice variant at the mRNA and protein levels in breast cancer cells. Alterations in the domain are of interest since it has been shown that the PAS/HLH domain can mediate homodimerization between family member and in this case may mediate crosstalk between nuclear receptors and PAS/HLH family members that have wide ranging effects in cellular growth control.

In this grant we have examined the hypothesis that the  $\Delta$  exon 3 splice AIB1 variant has an altered function, due to the removal of the PAS/HLH domain, which changes its interaction with nuclear receptors or other transcription regulators. We propose that changes in the level of

expression of  $\Delta$  exon 3 AIB1 will support tumor progression and will likely have prognostic significance for breast cancer.

## Body

### Statement of Work

#### ***Task 1. Analyze the function of the $\Delta$ exon 3 AIB1 isoform versus full-length AIB1 in transient transfection assays.***

We first compare the  $\Delta$  exon 3 AIB1 to the full length AIB1 protein with respect to their ability to potentiate nuclear receptor function in transient transfection assays in transformed and non-transformed breast cell lines.

We have now completed a series of experiments showing the  $\Delta$  exon 3 isoform is a significantly more effective transcriptional coactivator of both the estrogen receptor and the progesterone receptor. In addition, we have now shown that the  $\Delta$  exon 3 AIB1 variant potentiates epidermal growth factor signaling. The data we have obtained on the functional impact of the expression of  $\Delta$  exon 3 AIB1 in transient systems and the expression data showing that  $\Delta$  exon 3 mRNA is highly overexpressed in both breast cancer cell lines and breast tumor samples has now been published in a *J Biol Chem* paper and this manuscript can be found in the appendix of this report.

#### ***Task 2. Analyze the effect of stable expression of $\Delta$ exon 3 AIB1.***

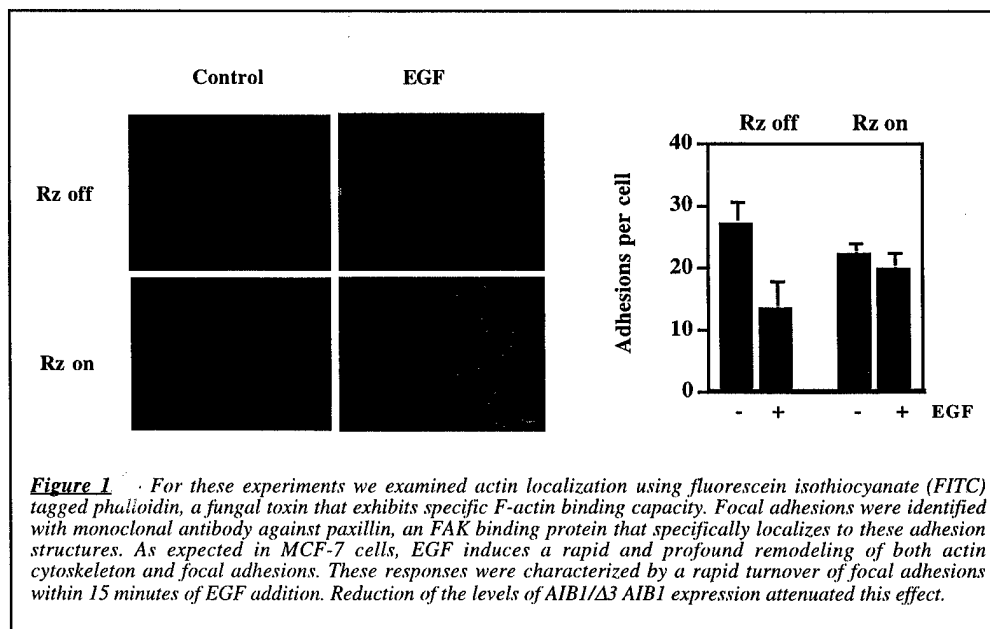
We will examine the impact of the expression of  $\Delta$  exon 3 AIB1 on normal breast epithelial and on breast cancer cell phenotype by developing cell lines stably expressing the  $\Delta$  exon 3 AIB1 or full-length AIB1. We will focus in particular on malignant transformation in the normal epithelial cells and on basal and hormone-induced proliferation and apoptosis in the cancer cells.

This task is currently in progress. We are trying to create cell lines that stably expressing the  $\Delta$  exon 3 and to help us with that we have now made a tagged version of the  $\Delta$  exon 3 expression vector both with a histidine tag at the C-terminus and also a MYC tag at the C-terminus to determine if we have cell lines that clearly overexpress  $\Delta$  exon 3. In the next year we will examine the phenotypic impact of overexpression of the  $\Delta$  exon 3 AIB1 variant vs. AIB1 in the tumorigenic phenotype. The phenotypic assays we will use were delineated in the original proposal.

#### ***Task 3. Determine the effect of overexpression of $\Delta$ exon 3 AIB1 on angiogenic and invasive properties of breast cancer cells.***

We will use cell lines developed under *Task 2* to determine the effects of  $\Delta$  exon 3 AIB1 on stromal and endothelial cell interactions with breast tumor cells.

We now have some preliminary data showing that reducing AIB1 in cells does impact on the invasive behavior of tumors. See Fig. 1.



For this we are encouraged that this  $\Delta$  exon 3 AIB1 splice variant will have a major impact on the angiogenic and invasive phenotype on MCF-7 cells and will continue to persevere with this task during the next year, using the cell lines we are currently developing under *Task 2*.

### Key Research Accomplishments

Task 1: Completed in MCF-7 cells. *J. Biol. Chem.* article attached.

Task 2: Underway

Task 3: Will be started as soon as cell lines available.

### Reportable Outcomes

#### Article

Reiter, R., Wellstein, A., and **Riegel, A.T.** "An Isoform of the Coactivator AIB1 that Increases Hormone and Growth Factor Sensitivity is Overexpressed in Breast Cancer." (2001) *J. Biol. Chem.* 276, 39736-39741.

#### Abstract

Reiter, R., Wellstein, A., and **Riegel, A.T.** "An Isoform of the Coactivator AIB1 that Increases Hormone and Growth Factor Sensitivity is Overexpressed in Breast Cancer." Poster presentation for the Era of Hope meeting, Orlando, FL, September 2002.

## **Patent**

“Coactivators in the Diagnosis and Treatment of Breast Cancer.” #RIAN432801 - Pending

## **Conclusions**

In this study we have provided evidence for the presence of a splice variant of AIB1 that has exon 3 deleted. The AIB1-Δ3 mRNA is translated in vivo in breast cancer cells into an NH2-terminal truncated form of AIB1 that has several properties of interest. The first is that on a per molecule basis it is a more potent transcriptional coactivator of both the estrogen and progesterone receptors than the full-length AIB1 protein. This result was unexpected given that previous studies of NH2-terminal deletion mutants of the AIB1-related protein SRC-1 did not reveal an impact on nuclear receptor signaling.

The second interesting aspect of the function of the AIB1-Δ3 isoform was that it also potently increased EGF signaling in ME-180 squamous carcinoma cells.

In addition, of major interest for breast cancer is our finding that the AIB1-Δ3 mRNA is overexpressed in breast cancer cell lines and in human breast tumors. Our analysis of tumor cell lines suggests that there is an overall increase in the AIB1-Δ3 mRNA relative to the full-length AIB1, although we do not know whether this is related to the gene amplification status of the endogenous gene.

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## An Isoform of the Coactivator AIB1 That Increases Hormone and Growth Factor Sensitivity Is Overexpressed in Breast Cancer\*

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The AIB1 (amplified in breast cancer 1) protein is a coactivator that potentiates the transcriptional activity of nuclear hormone receptors, and its gene is amplified in a subset of human breast cancers. Here we report a splice variant of *AIB1* mRNA that lacks the exon 3 sequence. We determined that the *AIB-Δ3* mRNA encoded a 130-kDa protein that lacks the NH<sub>2</sub>-terminal basic helix-loop-helix and a portion of the PAS (Per-Arnt-Sim homology) dimerization domain. The 130-kDa protein was detected in MCF-7 breast cancer cells at levels that were 5–10% of the full-length protein, whereas in non-transformed mammary epithelium lines, the AIB-Δ3 protein was present at significantly lower levels compared with the full-length AIB1. Consistent with this finding, the abundance of *AIB1-Δ3* mRNA was increased in human breast cancer specimens relative to that in normal breast tissue. To determine whether there were phenotypic changes associated with the overexpression of the AIB-Δ3 isoform, we performed functional reporter gene assays. These revealed that the ability of AIB1-Δ3 to promote transcription mediated by the estrogen or progesterone receptors was significantly greater than that of the full-length protein. Surprisingly, the AIB1-Δ3 isoform was also more effective than AIB1 in promoting transcription induced by epidermal growth factor. Overexpression of AIB1-Δ3 may thus play an important role in sensitizing breast tumor cells to hormone or growth factor stimulation.

Ligands such as estrogen and progesterone that interact with nuclear receptors regulate gene expression predominantly at the transcriptional level. The ligand-bound receptors interact specifically with DNA and activate transcription by recruiting a preinitiation complex. Although such gene activation was originally thought to be mediated by interaction of the receptors with components of the basal transcriptional machinery (1–6), a variety of screening techniques has identified a family of receptor-interacting proteins known as nuclear receptor coactivators (7–11). A common characteristic of this superfamily of proteins is that, when overexpressed in the presence of nuclear receptors, they potentiate ligand induction of transcription (12, 13). Members of the related p160 group of coactivators, which include steroid receptor coactivator 1 (SRC-1),<sup>1</sup>

SRC-2, and SRC-3 (also known as AIB1, ACTR, RAC3, TRAM-1, and p/CIP) (14–20), possess several similar structural features including a receptor interaction domain, a bHLH (basic helix-loop-helix)-PAS (Per-Arnt-Sim homology) dimerization domain, and a CBP interaction domain (13). Coactivators are thought to function as bridges between nuclear receptors and either other coactivators or the basal transcriptional machinery (13). However, the discovery that coactivators possess a histone acetylase domain (15, 21–24) suggests that these proteins also might serve to regulate chromatin structure.

A portion of human chromosome 20q that is frequently amplified in breast cancer contains the gene for the nuclear coactivator AIB1 (amplified in breast cancer 1) (25). The *AIB1* gene is amplified in 5–10% of breast cancers, and the abundance of the corresponding mRNA and protein is increased in some breast tumors and breast cancer cell lines (14, 25–27). It has recently been shown that AIB1 binds directly to the estrogen receptor (ER) (28) and that AIB1 is rate-limiting for estrogen-induced growth of MCF-7 cells (29). However, the overall role of AIB1 for breast tumorigenesis is not clear because AIB1 potentiates not only the action of estrogen (14, 16) and progesterone (16) receptors but also that of various other nuclear receptors (9, 15, 17–20) and transcription factors (30, 31). In addition, several splice variants of SRC family members have been described, although the functions of these variants remain unknown (13).

Here we report the identification of a splice variant of *AIB1* that is overexpressed in breast cancer tissue and cell lines. The *AIB-Δ3* transcript encodes an NH<sub>2</sub>-terminal truncated version of AIB1 that lacks the bHLH and PAS A domains. In functional studies we have determined that the AIB-Δ3 protein is a significantly more effective coactivator of estrogen, progesterone, and EGF signaling than the wild type ER, suggesting a role for this AIB1 isoform in hormone and paracrine signaling in breast cancer.

### EXPERIMENTAL PROCEDURES

**Plasmids**—We subcloned the full-length *AIB1* cDNA from pCMX-ACTR (provided by R. Evans, Salk Institute, La Jolla, CA) into pcDNA3 (Invitrogen) with the use of the flanking *KpnI* and *XhoI* sites, thereby creating the expression vector pcDNA3-AIB1. We subcloned the smaller of the two RT-PCR products generated from MCF-7 cell total RNA with exon 1- and exon 9-specific primers (Fig. 1b) into pCRII (Invitrogen). The resulting plasmid was digested with *BamHI* and *HpaI*, recognition sequences that flank the splice sites of *AIB1-Δ3* cDNA, and the released fragment was purified and used to replace the corresponding sequence of pcDNA3-AIB1, thereby creating pcDNA3-AIB1-Δ3. The pcDNA3-

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<sup>1</sup> The abbreviations used are: SRC, steroid receptor coactivator;

bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim homology; ER, estrogen receptor; ERE, estrogen response element; PCR, polymerase chain reaction; RT, reverse transcription; IMEM, Iscove's modified Eagle's medium; FBS, fetal bovine serum; EGF, epidermal growth factor; CHO, Chinese hamster ovary; FGF-BP, fibroblast growth factor-binding protein; TEF, transcription-enhancing factor.

AIB1 and pcDNA3-AIB1-Δ3 vectors contain identical 5'- and 3'-untranslated regions, differing only in the loss of exon 3 in the latter. The inserts were verified by sequencing.

The expression vectors for human estrogen receptor  $\alpha$  and progesterone receptor B were provided by P. Chambon (CNRS, France). The firefly luciferase reporter plasmid containing the estrogen response element (ERE) from the *Xenopus* vitellogenin gene was provided by V. C. Jordan (Northwestern University, Chicago), and the plasmid containing the mouse mammary tumor virus promoter was provided by G. Hager (National Cancer Institute, Bethesda, MD). The luciferase reporter plasmid containing the human *FGF-BP* gene promoter has been described previously (32). The *Renilla* luciferase vector (pRL-CMV) was from Promega.

**Cells and Tissue Samples**—All cell lines were obtained from the tissue culture core facility of the Lombardi Cancer Center. MCF-7, ME-180, and COS-1 cells were cultured in Iscove's modified Eagle's medium (IMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). MCF-10A and A1N4 cells were grown in a 1:1 mixture of IMEM and Ham's F-12 medium (Life Technologies, Inc.) that was supplemented with 5% horse serum, EGF (20 ng/ml), insulin (10  $\mu$ g/ml), and hydrocortisone (500 ng/ml). CHO cells were maintained in F-12 nutrient mixture (Life Technologies, Inc.) supplemented with 10% FBS.

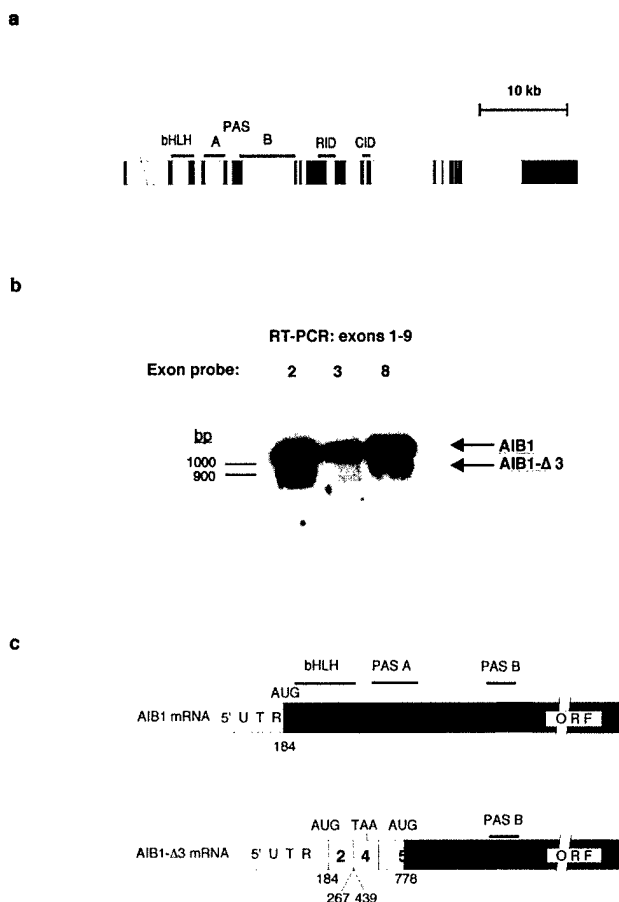
Frozen tissue samples were obtained from the Lombardi Cancer Center Histopathology and Tissue Shared Resource Core. The six normal samples were obtained from individuals undergoing reduction mammoplasty (mean age at time of surgery, 29 years; range, 19 to 54 years); the eight primary breast carcinoma specimens were obtained from women with a mean age at the time of surgery of 51 years (range, 29 to 64 years).

**Immunoblot Analysis**—Whole cell extracts were prepared as described previously (32), and equal portions (30  $\mu$ g of protein) were resolved either on denaturing 4–20% polyacrylamide gradient gels or on 4% polyacrylamide gels containing Tris-glycine. The separated proteins were transferred to a nitrocellulose membrane and then subjected to immunoblot analysis with a 1:500 dilution of a mouse monoclonal antibody specific for amino acids 376–389 of human AIB1 (Transduction Laboratories), horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin (1:10,000 dilution; Amersham Pharmacia Biotech), and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

**RT-PCR**—Isolation of total RNA and synthesis of cDNA by RT were performed as described previously (33). The amplification of AIB1 cDNA sequences was achieved by PCR according to the following protocol: incubation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 90 s. The oligonucleotides used as primers for PCR or as probes for hybridization were as follows: exon 1, 5'-GACTGGTTAGCCAGTTGCTG-3'; exon 2, 5'-GCCATGTGATCTC-CAGGAC-3'; exon 3, 5'-CTGAGCTGATATCTGCCAATC-3'; exon 4, 5'-AGCCGATGTATCTTCTACAGG-3'; exon 5, 5'-ATGTTTCCGTCTCGA-TTACC-3'; exon 8, 5'-CCTCATGGAGATCTCAGT-3'; and exon 9, 5'-CCATCAGCCAACGAGAATCG-3'. The PCR products were separated by electrophoresis on a 1% agarose gel, transferred to a polyvinylidene difluoride membrane, and hybridized with a <sup>32</sup>P-labeled oligonucleotide probe. Quantification of PCR products was performed with a PhosphorImager (Molecular Dynamics 445SI).

**Transient Transfection and Reporter Gene Assays**—COS-1 and CHO cells were plated at densities of  $2 \times 10^5$  and  $5 \times 10^5$  cells/well, respectively, in six-well plates and were cultured for 24 h at 37 °C under 5% CO<sub>2</sub> in IMEM or Ham's F-12, respectively, supplemented with 5% FBS that had been treated with dextran-coated charcoal. The medium was then replaced with IMEM containing LipofectAMINE Plus (Life Technologies, Inc.) and expression and reporter plasmids as indicated. After incubation for 3 h, the medium was replaced with IMEM (COS-1 cells) or Ham's F-12 (CHO cells), each containing 5% dextran-coated charcoal-treated FBS and nuclear receptor ligands. Cells were incubated for 24 h and then disrupted in passive lysis buffer (Promega). Portions (20  $\mu$ l) of the resulting cell extract were assayed for both firefly and *Renilla* luciferase activities with the Dual-Luciferase reporter assay system (Promega).

ME-180 cells were plated at a density of  $5 \times 10^5$  cells/well and cultured for 24 h in IMEM supplemented with 5% dextran-charcoal-treated FBS. They were then incubated for 3 h in IMEM supplemented with LipofectAMINE Plus and expression and reporter plasmids. The cells were washed and then incubated in IMEM for an additional 3 h before incubation for 18 h with EGF (5 ng/ml) in serum-free IMEM and subsequent lysis. Because of high background induction of pRL-CMV expression by EGF, firefly luciferase activity was normalized by protein



**FIG. 1. Characterization of a splice variant of human AIB1.** *a*, structure of human AIB1 showing the 22 known exons (filled boxes) and the corresponding introns (open boxes). The exon/intron regions, which are spliced to form the various functional domains of the AIB1 protein, are indicated by horizontal bars. *b*, detection of the AIB1-Δ3 splice variant in total RNA from MCF-7 cells. The total RNA was subjected to RT-PCR with primers specific for exons 1 and 9 of AIB1. The reaction products were resolved on a 1% agarose gel and transferred to a polyvinylidene difluoride membrane, which was then cut, and the lanes were subjected separately to hybridization with <sup>32</sup>P-labeled oligonucleotides specific for exons 2, 3, or 8 of AIB1. The positions of PCR products corresponding to the full-length (AIB1) and truncated (AIB1-Δ3) transcripts are indicated. *bp*, base pairs. *c*, comparison of the structures of AIB1 and AIB1-Δ3 mRNAs. The alternative splicing event that results in the loss of exon 3 causes the open reading frame (ORF) to shift and terminate at a TAA codon in exon 4. A potential initiation site (AUG) for AIB1-Δ3 mRNA is present at nucleotide 778; the use of this site would be consistent with the AIB-Δ3 protein lacking the NH<sub>2</sub>-terminal 26 kDa of full-length AIB1. The shaded regions indicate the open reading frame, and exons in the mRNAs are numbered. The positions of the splice junctions in AIB1-Δ3 mRNA and of the encoded protein domains are indicated. UTR, untranslated region.

concentration as described previously (32).

**In Vitro Transcription-Translation**—*In vitro* transcription-translation was performed with the TnT coupled reticulocyte lysate system (Promega). Plasmid DNA (1  $\mu$ g) was combined with 25  $\mu$ l of rabbit reticulocyte lysate, 2  $\mu$ l of TnT reaction buffer, 1  $\mu$ l of T7 RNA polymerase, 1  $\mu$ l of amino acid mixture, 1  $\mu$ l of Rnasin (Ambion) ribonuclease inhibitor (40 units), and 1  $\mu$ l of Transcend biotin-lysyl-tRNA (Roche) and the final volume was adjusted to 50  $\mu$ l. The reaction was performed at 30 °C for 90 min, after which 5  $\mu$ l of the reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis and either to immunoblot analysis with antibodies to AIB1 or to direct detection with streptavidin-conjugated horseradish peroxidase (1:10,000 dilution in phosphate-buffered saline containing 0.05% Tween 20) and enhanced chemiluminescence.

## RESULTS

**Detection of the AIB1-Δ3 Isoform**—In this study we determined whether there are naturally occurring splice variants of

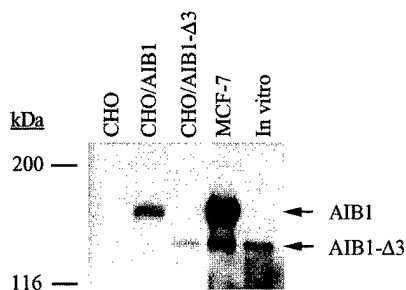


FIG. 2. Immunoblot analysis of AIB1 isoforms in extracts of MCF-7 cells and transfected CHO cells. Extracts of untransfected CHO cells or CHO cells transfected with plasmids encoding AIB1 or AIB1- $\Delta$ 3, or MCF-7 cells were fractionated by electrophoresis on high resolution 4% polyacrylamide gels containing Tris-glycine, and the separated proteins were transferred to a nitrocellulose membrane and probed with a monoclonal antibody specific for amino acids 376–389 of human AIB1. The products of *in vitro* transcription-translation of AIB1- $\Delta$ 3 cDNA were similarly analyzed.

AIB1 present in breast cancer cells that might encode proteins with altered function relevant to breast cancer progression. The exon-intron structure of AIB1 was assembled as shown in Fig. 1a by comparing the published sequence of the cDNA (14) with the contiguous genomic sequence available through the NCBI data base. We arbitrarily designated the most 5' exon of AIB1 as exon 1, with the result that the first codon is located in exon 2. Our initial strategy was to determine whether RNA from MCF-7 cells, which overexpress AIB1 (14), contained any splice variant forms of AIB1 RNA. To achieve this end, we performed reverse transcription and polymerase chain reaction (RT-PCR) analysis of total RNA from MCF-7 human breast cancer cells with primers amplifying the region between exons 1 and 9. This revealed two PCR products that differed in size by ~150 base pairs. These PCR products were then subjected to Southern blot analysis, and individual lanes from the membrane were probed separately with oligonucleotides specific for each exon from 2 to 8. Typical hybridizations with exons 2, 3, and 8 are shown in Fig. 1b. This analysis revealed that the smaller PCR product hybridized with all probes except the one specific for exon 3 (Fig. 1b), indicating that the lower band corresponds to an RNA splice variant (designated AIB1- $\Delta$ 3) of AIB1 that lacks the exon 3 sequence. We subsequently subcloned and sequenced this PCR product, confirming that nucleotides 267–439 (exon 3) of the full-length AIB1 cDNA were missing (Fig. 1c).

**Translation of the AIB1- $\Delta$ 3 mRNA in Vitro and in Vivo**—To determine whether an AIB1-related protein was encoded by the AIB1- $\Delta$ 3 mRNA, we performed *in vitro* transcription and translation of AIB1- $\Delta$ 3 cDNA. Western blot analysis with an AIB1-specific antibody of the proteins translated *in vitro* revealed the production of a 130-kDa protein (Fig. 2). Interestingly, we had also consistently detected a similar 130-kDa protein, in addition to the 156-kDa full-length AIB1, by immunoblot analysis of MCF-7 cell extracts with antibodies to AIB1 on 5–20% polyacrylamide gels (27). To determine whether the MCF-7 130-kDa species and the *in vitro* transcription translation product had identical electrophoretic properties, we performed high resolution electrophoresis on 4% polyacrylamide gels containing Tris-glycine followed by immunoblot analysis. This analysis demonstrated that the mobility of the 130-kDa protein detected in MCF-7 cell extracts was identical to that of the 130-kDa protein produced by *in vitro* transcription and translation of AIB1- $\Delta$ 3 cDNA (Fig. 2). This observation suggested that the 130-kDa MCF-7 cell protein was translated from AIB1- $\Delta$ 3 mRNA present in these cells.

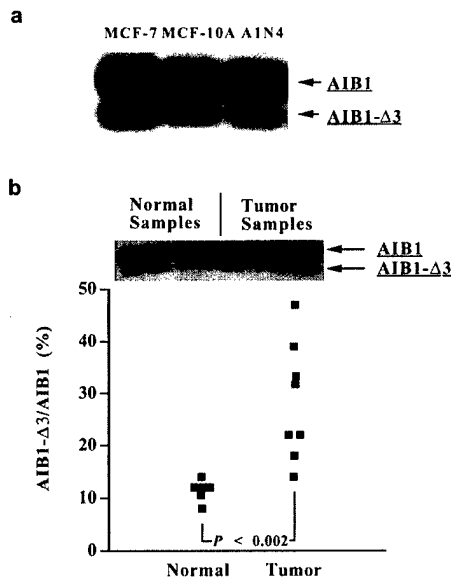
To verify that the AIB1- $\Delta$ 3 mRNA was translated *in vivo* we performed transient transfection of CHO cells (Fig. 2; see Fig.

4a) or COS-1 cells (see Fig. 5a) with the AIB1- $\Delta$ 3 cDNA. Analysis of cell extracts demonstrated that this indeed resulted in the production of a 130-kDa protein, whereas transfection with the full-length AIB1 cDNA yielded only the 156-kDa full-length protein. This latter observation demonstrated that the 130-kDa protein was clearly not the product of proteolytic processing of the full-length protein. The electrophoretic mobility of the 130-kDa protein synthesized in cells transfected with the AIB1- $\Delta$ 3 cDNA was identical to that of both the 130-kDa AIB1 species present in MCF-7 cell extracts and the product of *in vitro* transcription-translation of the AIB1- $\Delta$ 3 cDNA (Fig. 2). Together these data indicated that the endogenous AIB1- $\Delta$ 3 mRNA present in MCF-7 cells encodes a 130-kDa protein.

An examination of the sequence of AIB1- $\Delta$ 3 mRNA indicated that the open reading frame of AIB1, which initiates at nucleotide 184 in the full-length mRNA would terminate after 90 amino acids in the splice variant (Fig. 1c). We did not detect this predicted low molecular mass product *in vivo* or *in vitro*. The 130-kDa species is detected by an AIB1 antibody raised against amino acids 376–389 in the amino terminus of the protein. This suggests that the AIB1- $\Delta$ 3 isoform most likely represents an NH<sub>2</sub>-terminally truncated form of AIB1, with synthesis being initiated at an internal translation start site downstream of the splice junction but prior to amino acid 376. Such internal translational initiation has been described for various mRNAs with extended 5'-untranslated regions (34–37). The difference in size between the 156-kDa full-length AIB1 protein and the 130-kDa species suggested that the latter lacks ~210 amino acids of the former, including all of the bHLH region (residues 16–88) and most of the PAS A domain (residues 116–171) (Fig. 1c). This would place the initiation codon for the 130-kDa protein most likely at the codon at position 778 (Fig. 1c). Interestingly, for cells transfected with equivalent amounts of cDNA, the intracellular concentration of AIB1- $\Delta$ 3 protein was ~10% of that of full-length AIB1 (Fig. 2; see Figs. 4a and 5a), suggesting that translation initiation of the splice variant was inefficient, possibly because of the long 5'-untranslated region of the AIB1- $\Delta$ 3 mRNA.

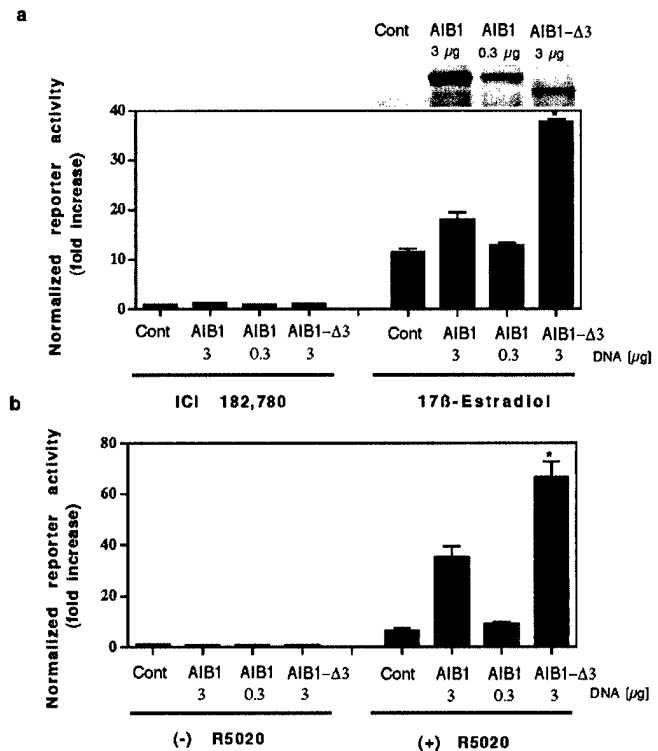
**AIB1- $\Delta$ 3 mRNA Is Overexpressed in Human Breast Cancer**—Given that we first detected the AIB1- $\Delta$ 3 splice variant in a breast cancer cell line, we next examined whether its expression was restricted to tumor cells. MCF-7 cells are derived from a pleural effusion of metastatic breast cancer, whereas MCF-10A and A1N4 cells are not malignantly transformed and were derived from atypical human breast epithelial hyperplasia (38) and from human mammary epithelial cells treated with benzo[a]pyrene (39), respectively. RT-PCR followed by Southern blot analysis revealed that the amounts of AIB1- $\Delta$ 3 mRNA in MCF-10A and A1N4 cells were lower than that of MCF-7 cells (Fig. 3a). By subsequent real-time PCR analysis, using primers specific for AIB1 or its isoform, we have assessed that the ratio of AIB1- $\Delta$ 3 mRNA/full-length AIB1 is 5% in MCF-7 cells, whereas in MCF-10A and A1N4 cells the ratio is 0.5% (data not shown). We then compared the abundance of the AIB1- $\Delta$ 3 mRNA in a series of eight human breast tumors with that in normal tissue obtained from six women undergoing breast reduction mastoplasty. The amount of the full-length AIB1 mRNA in tumor samples was slightly greater than that in the normal tissue samples, but this difference was not significant (Fig. 3b). In contrast, the abundance of the AIB1- $\Delta$ 3 mRNA in the tumor specimens was significantly greater than that in the normal tissue samples, with all but one of the tumors showing an increased amount of this transcript compared with the normal range.

**Effect of the AIB1- $\Delta$ 3 Isoform on Nuclear Receptor Function**—We next examined the effect of the deletion of the bHLH



**FIG. 3. Comparison of the abundance of AIB1-Δ3 mRNA in malignant and nonmalignant human breast tissue and cell lines.** *a*, total RNA isolated from MCF-7, MCF-10A, and A1N4 cells was subjected to RT-PCR with primers specific for exons 2 and 5 of AIB1. The reaction products were resolved on a 1% agarose gel and then subjected to Southern blot analysis with a  $^{32}$ P-labeled oligonucleotide probe specific for exon 4 of AIB1. *b*, total RNA isolated from six normal breast and eight breast cancer tissue samples was analyzed as in panel *a*. The amounts of PCR products corresponding to AIB1 and AIB1-Δ3 mRNAs were quantitated by densitometry, and the abundance of the latter was expressed as a percentage of that of the former. The signal of the full-length AIB1 transcript was compared between breast tumors and normal breast tissue with the use of an arbitrary scale; the signals in tumor and normal samples were  $1.0 \pm 0.46$  and  $0.7 \pm 0.24$  (means  $\pm$  S.E.), respectively, and they did not differ significantly ( $p > 0.05$ ; Student's *t* test). The inset shows a typical blot of 8 of the 14 samples.

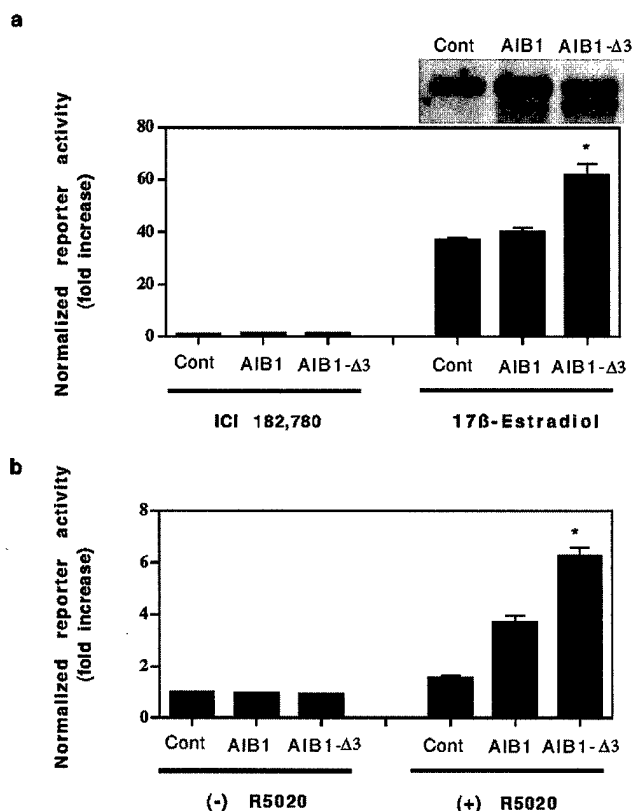
and PAS A domains in AIB1-Δ3 on protein function. AIB1 acts as a coactivator for several nuclear receptors, including those for estrogen and progesterone, which are important in breast carcinogenesis. We therefore transfected CHO cells with expression vectors encoding full-length AIB1 or AIB1-Δ3, an expression vector for estrogen receptor  $\alpha$ , and a luciferase reporter plasmid containing an ERE. Transfection of CHO cells with 3  $\mu$ g of the AIB1 expression vector resulted in a 1.4-fold increase in estrogen-induced luciferase activity, whereas transfection with 3  $\mu$ g of the vector for AIB1-Δ3 resulted in a 3.8-fold increase in the estrogen response (Fig. 4*a*). However, given that the abundance of recombinant AIB1 in the transfected cells was about 10 times that of AIB1-Δ3, we also transfected CHO cells with 0.3  $\mu$ g of the AIB1 vector, which yielded about the same amount of intracellular recombinant protein as did 3  $\mu$ g of the AIB1-Δ3 vector (Fig. 4*a*). A comparison of transfected cells containing approximately equal amounts of recombinant protein thus revealed that AIB1 and AIB1-Δ3 potentiated the estrogen response by factors of 1.1 and 3.8, respectively. Similar transfection experiments with COS-1 cells (which express endogenous AIB1) also demonstrated a greater potentiation of the estrogen response by AIB1-Δ3 than by full-length AIB1 (Fig. 5*a*). The differences between full-length and the AIB1-Δ3 isoform were seen at different concentrations of estrogen (0.1–10 nM) and thus were not due to a change in the affinity of the hormone for its receptor but rather suggest enhanced efficacy of the signaling (data not shown). We also obtained similar results in COS-1 cells with an expression vector encoding progesterone receptor B; the transcriptional response to the progesterone analog R5020 was thus potentiated to a greater extent by AIB1-Δ3 than by AIB1 in both CHO and COS-1 cells (Figs. 4*b* and 5*b*). Of particular note is that small amounts of



**FIG. 4. Effects of AIB1 and AIB1-Δ3 on the activation of estrogen receptor  $\alpha$  and progesterone receptor B in CHO cells.** *a*, cells were transfected with either the empty pcDNA3 vector (3  $\mu$ g) (Cont), pcDNA3-AIB1 (0.3 or 3  $\mu$ g), or pcDNA3-AIB1-Δ3 (3  $\mu$ g) together with an expression vector for human estrogen receptor  $\alpha$  (100 ng), an ERE-luciferase reporter plasmid (1  $\mu$ g), and pRL-CMV (0.1 ng). After incubation for 24 h with either 10 nM 17 $\beta$ -estradiol or 100 nM estrogen receptor antagonist ICI 182,780, cells were lysed and assayed for luciferase activity. The inset shows immunoblot analysis of transfected cell lysates that were fractionated on 4–20% polyacrylamide gradient gels and probed with antibodies to AIB1. *b*, cells were transfected as in panel *a* with the exception that the estrogen receptor vector was replaced with a vector for human progesterone receptor B (20 ng) and the ERE-luciferase plasmid was replaced by a luciferase reporter construct containing the mouse mammary tumor virus promoter (2  $\mu$ g). Cells were incubated for 24 h in the absence or presence of the progesterone analog R5020 (1 nM) before preparation of lysates for luciferase assay. The firefly luciferase activity of cell lysates was divided by the *Renilla* luciferase activity (internal control), and this ratio (normalized reporter activity) for control cells incubated in the absence of agonist was assigned a value of 1. Data are means  $\pm$  S.E. of values from three independent experiments, each performed in triplicate. \*,  $p < 0.005$  versus corresponding value for cells transfected with 3  $\mu$ g of the AIB1 vector (Student's *t* test).

transfected AIB1-Δ3 protein had significant effects on ER- and progesterone receptor-induced transcription even against a relatively high background of full-length AIB1 (Fig. 5, *a* and *b*).

**Effect of the AIB1-Δ3 Isoform on EGF Signaling**—The fact that members of the p160 SRC family act as coactivators in intracellular signaling pathways that activate transcription factors other than nuclear receptors (30, 31) prompted us to examine whether AIB1-Δ3 might be able to sensitize breast cancer cells to growth factor signaling. Overexpression of members of the families of epidermal growth factor (EGF) ligands or EGF receptors is important in the malignant progression of breast cancer (40). Such growth factors also contribute to the hormone-independent phenotype of breast tumors and the HER-2 receptor is a target of current therapies (41). To determine whether AIB1 and AIB1-Δ3 affect EGF signaling, we transfected ME-180 human squamous cell carcinoma cells with the respective expression vectors and with a luciferase reporter plasmid containing the promoter of the fibroblast growth factor-binding protein (FGF-BP) gene. FGF-BP functions as an

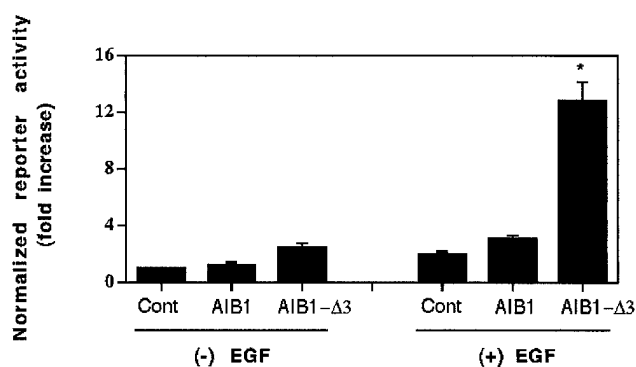


**FIG. 5. Effects of AIB1 and AIB1-Δ3 on the activation of estrogen receptor  $\alpha$  and progesterone receptor B in COS-1 cells.** *a*, cells were transfected and analyzed as described in the legend for Fig. 4a (the amount of pcDNA3-AIB1 was 3  $\mu$ g). *b*, cells were transfected with 1  $\mu$ g of either pcDNA3, pcDNA3-AIB1, or pcDNA3-AIB1-Δ3 together with an expression vector for human progesterone receptor B (10 ng), a luciferase reporter plasmid containing the mouse mammary tumor virus promoter (1  $\mu$ g), and pRL-CMV (0.1 ng). After incubation for 24 h in the absence or presence of 0.5 nM R5020, cells were lysed and assayed for luciferase activity. Data are means  $\pm$  S.E. of values from three independent experiments, each performed in triplicate. \*,  $p < 0.005$  versus the corresponding value for cells transfected with the AIB1 vector.

angiogenic switch molecule (42) that is overexpressed in breast cancer, and its gene is activated by EGF in squamous cell and breast cancer cell lines (32). As reported previously, EGF induced a 2.5-fold increase in reporter activity in control cells transfected with the empty expression vector (Fig. 6). The basal EGF induction was increased slightly by transfection of the full-length AIB1 expression vector, whereas EGF induction was increased ~6-fold by expressing recombinant AIB1-Δ3.

#### DISCUSSION

In this study we have provided evidence for the presence of a splice variant of AIB1 that has exon 3 deleted. The AIB1-Δ3 mRNA is translated *in vivo* in breast cancer cells into an NH<sub>2</sub>-terminal truncated form of AIB1 that has several properties of interest. The first is that on a per molecule basis it is a more potent transcriptional coactivator of both the estrogen and progesterone receptors than the full-length AIB1 protein. This result was unexpected given that previous studies of NH<sub>2</sub>-terminal deletion mutants of the AIB1-related protein SRC-1 did not reveal an impact of this region on nuclear receptor signaling (9, 30). One possible reason for the increased activity of AIB1-Δ3 is that the conformation of this isoform is more favorable than that of the full-length protein for interaction with nuclear receptors or for recruitment of other coactivators such as CBP/p300. An alternative possibility is suggested by the observation that the bHLH-PAS domain of SRC-1 interacts



**FIG. 6. Effects of AIB1 and AIB1-Δ3 on activation of the FGF-BP gene promoter by EGF in ME-180 cells.** Cells were transfected with 3  $\mu$ g of either pcDNA3, pcDNA3-AIB1, or pcDNA3-AIB1-Δ3 together with a luciferase reporter plasmid containing the human FGF-BP gene promoter (1  $\mu$ g). After incubation of cells for 18 h in the absence or presence of EGF (5 ng/ml) in serum-free medium, cell extracts were prepared and assayed for luciferase activity. Activity was normalized by protein concentration, and the normalized activity values were then expressed relative to that of cells transfected with pcDNA3 and not exposed to EGF. Data are means  $\pm$  S.E. of values from three independent experiments, each performed in triplicate. \*,  $p < 0.01$  versus the corresponding value for control cells.

with and potentiate the activity of members of the TEF (transcription-enhancing factor) family of transcription factors (30). Thus, full-length AIB1 might be unavailable for interaction with nuclear receptors because it is sequestered or squelched by other intracellular factors. In contrast, AIB1-Δ3, which lacks an intact bHLH-PAS domain, would not bind to factors such as TEF and would be available for nuclear receptor coactivation. This notion might explain why relatively small amounts of recombinant AIB1-Δ3 are able to induce significant potentiation of nuclear receptor activity in transfected COS-1 cells with a high background of endogenous full-length AIB1. This model also predicts that the relative coactivating effects of AIB1 and AIB1-Δ3 might be cell type-specific, depending on the endogenous expression of AIB1-sequestering molecules such as TEF. Interestingly, a recent report has described that the human MMS19 protein can interact with the PAS-HLH domain of AIB1 and can regulate ER-mediated transcriptional activity (43). It is possible that the lack of interaction of AIB1-Δ3 with this protein may explain some of its increased effectiveness *in vivo*. Whatever the reason for the increased activity of the AIB1-Δ3 isoform, our data suggest that its expression would sensitize cells to the effects of estrogen and progesterone.

The second interesting aspect of the function of the AIB1-Δ3 isoform was that it also potentially increased EGF signaling in ME-180 squamous carcinoma cells. This could be through direct interactions with a nuclear receptor. However, our analysis of the fragment of the FGF-BP gene promoter (nucleotides -118 to +62, relative to the transcription start site) used in this study did not reveal obvious consensus recognition sites for known nuclear receptors. In fact, EGF induction of this promoter is dependent on the factors AP-1 and c/EBP $\beta$  (32), either of which may interact directly or indirectly with AIB1. Alternatively it may be that a common intermediary of both nuclear receptor and AP-1 signaling such as CBP/p300 (44, 45) may be the target of the superactivating effects of the AIB1-Δ3 isoform. Whatever the mechanism of the increased potentiation of growth factor signaling by the AIB1-Δ3 isoform, our data suggest that an increase in the abundance of the AIB1-Δ3 isoform in mammary epithelial cells may be an important step in tumor progression and in the development of a more aggressive, hormone-independent phenotype.

Finally, of major interest for breast cancer is our finding that the AIB1-Δ3 mRNA is overexpressed in breast cancer cell lines

and in human breast tumors. Our analysis of tumor cell lines suggests that there is an overall increase in the AIB1- $\Delta 3$  mRNA relative to the full-length AIB1, although we do not know whether this is related to the gene amplification status of the endogenous gene. Alternatively, the increase in AIB1- $\Delta 3$  mRNA may be because of an increase in RNA splicing of exon 3 in breast cancer cells. It is also possible that the increase in expression in tumors may be due in part to dilution effects of surrounding stromal tissue, but this seems unlikely given that we also see lower AIB1- $\Delta 3$  mRNA expression in non-transformed *versus* malignant mammary epithelial cell lines. To date a number of laboratories, including ours, have reported overexpression of AIB1 mRNA and protein in breast tumor tissue, although the assessment of the portion of breast cancers overexpressing AIB1 varies widely between groups (14, 27, 46, 47). In addition, some groups have determined that AIB1 overexpression is correlated with ER and progesterone receptor status (26), whereas others have found an inverse relationship with steroid receptor expression but a positive correlation with HER-2 and p53 expression (47). However, all of these RT-PCR or immunohistochemical analyses of expression levels have not distinguished the AIB1- $\Delta 3$  isoform signal from that of the wild type. Our data indicates that the overexpression of relatively low levels of the AIB1- $\Delta 3$  isoform can sensitize cells to estrogen, progesterone, and growth factors. Therefore we believe that measurement of increased levels of AIB1- $\Delta 3$  levels might be a sensitive indicator of the progression of breast cancer to a more hormone-independent phenotype.

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